Diabetologia (1997) 40: 302–306

Diabetologia

Amiloride-sensitive Na^+/H^+ exchange in erythrocytes of patients with NIDDM: a prospective study

W. Koren[1], R. Koldanov[1], V.S.Pronin[2], I.Y. Postnov[3], E. Peleg[1], T. Rosenthal[1], M. Berezin[4], Y.V.Postnov[3]

[1] Hypertension Unit, Chaim Sheba Medical Center, Tel Hashomer, Israel
[2] Institute of Endocrinology, Moscow, Russia
[3] Ministry of Health Central Research Laboratory, Moscow, Russia
[4] Endocrinology Institute, Chaim Sheba Medical Center, Tel Hashomer, Israel

Summary Intensive treatment of non-insulin-dependent diabetes mellitus (NIDDM) decreases the rate of microvascular complications, but is associated with increased incidence of cardiovascular morbidity. Enhanced permeability of plasma membranes for sodium (e.g. sodium-hydrogen exchange, NHE) may predict the subset of diabetic patients for whom intensive modalities of treatment are indicated despite their potential risk. However, the accuracy of NHE as a marker of microangiopathy has not been assessed. In this study NHE as initial velocity of amiloride-inhibited H^+ efflux from erythrocytes (pH_i 6.35–6.45) into an Na^+-containing medium (pH_o 7.95–8.05), was estimated during 8 years of follow-up in 138 non-microalbuminuric diabetic patients (74 women, 64 men, age 52 ± 4 years) treated with antihyperglycaemic drugs for 14 ± 2 years. Appearance of microalbuminuria, overt proteinuria, azotaemia and retinopathy was assessed annually. Enhanced erythrocyte NHE predicted diabetic nephropathy alone and in association with a family history of hypertension and/or nephropathy with a sensitivity of 86 and 93 %, respectively. No association was found between NHE and retinopathy in NIDDM. It is concluded that assessment of erythrocyte NHE can identify a subset of patients likely to develop renal damage, for whom an aggressive treatment approach might be considered. [Diabetologia (1997) 40: 302–306]

Keywords Na^+/H^+ exchange, NIDDM, diabetic nephropathy, diabetic retinopathy, erythrocytes.

Microangiopathy disables thousands of diabetic patients throughout the world. Aggressive treatment modalities, such as combined therapy with antihyperglycaemic drugs and insulin, appear to decrease the extent of microvascular damage, but are associated with increased incidence of hypoglycaemic events and weight gain [1]. Their risk-to-benefit ratio is high also because less than one third of diabetic patients develop nephropathy [2].

Received: 14 June 1996 and in final revised form: 8 November 1996

Corresponding author: W. Koren, M.D., Department of Medicine C, Chaim Sheba Medical Center, Tel Hashomer 52621, Israel

Abbreviations: NHE, Sodium-hydrogen exchange; NIDDM, non-insulin-dependent diabetes mellitus; MAP, mean arterial pressure.

Attempts have been made to find a marker that can predict micro-angiopathy in diabetes, and identify a group of patients for whom aggressive treatment is indicated despite its possible adverse effects. Urinary excretion of very small amounts of albumin (microalbuminuria) and elevation of arterial pressure [3] have been proposed for this purpose, but both appear when renal damage is already impending [4].

Enhanced permeability of extracellular membranes to sodium (defined as sodium-lithium and sodium-hydrogen antiports) found in blood cells and skin fibroblasts of diabetic patients prone to renal damage [5–7] is, however, a very early (if not congenital) sign. To assess the reliability of Na^+/H^+ exchange (NHE) study in predicting diabetic nephropathy, we followed initially non-microalbuminuric patients with non-insulin-dependent diabetes mellitus (NIDDM) of at least 10 years' duration for 8 years after NHE evaluation was first made.

Subjects and methods

The initial study group consisted of 164 patients (81 women and 83 men), aged 40–70 years, with maturity onset NIDDM. The criteria for enrollment were: at least 10 years duration of diabetes treatment, absence of proteinuria/microalbuminuria, normal arterial blood pressure, normal serum urea, creatinine and electrolyte levels, and absence of clinically evident retinopathy at the time of enrollment. Anamnestic or clinical evidence of recurrent or chronic urinary infection was the single exclusion criterion both on enrollment and during follow-up. Informed consent was obtained from all patients who participated in the study, which was conducted in accordance with the principles of the Declaration of Helsinki.

The diagnosis of diabetes was revised according to international criteria [8]. The patients were asked about their first-and-second degree hypertensive relatives. Blood pressure was measured as recommended by Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure [9]. Glycaemic control was estimated by plasma glucose and HbA_1 levels. Diastolic blood pressure was defined by disappearance of the Korotkoff sounds (phase V). Established hypertension was defined as the mean of three different blood pressure measurements above 160/95 mm Hg. Mean arterial pressure (MAP) was calculated as 1/3 (systolic + 2 × diastolic blood pressure). Elevated MAP was defined as MAP over 115 mm Hg.

Measurements were made of serum urea, creatinine and electrolytes. Urinary albumin secretion was determined by a solid-phase fluoroimmunoassay [10]. Urinary excretion of albumin between 20 and 300 mg/24 h was defined as microalbuminuria. Urinary excretion of albumin exceeding 300 mg/24 h was defined as overt proteinuria. Creatinine of 132.5 μmol/l or more was defined as azotaemia. Patients with azotaemia and/or overt proteinuria were concluded as having nephropathy.

After enrollment the patients were examined annually for 8 years, during which blood glucose, HbA_1,serum urea and creatinine, and urine protein/microalbumin were measured. Diabetic complications during the previous year were registered, including hypoglycaemic episodes, weight gain and ketosis. The patients were also examined by an ophthalmologist.

Twenty patients, 7 men and 13 women, were lost to follow-up. Six women were excluded from the study after they developed recurrent pyelonephritis. The remaining study group included 138 patients, 64 men and 74 women, aged 52 ± 4 years, with diabetes treated with antihyperglycaemic drugs for 14 ± 2 years.

Patients who developed overt proteinuria or azotaemia underwent ultrasound examination of their kidneys. The triad of enlarged kidneys, proteinuria and long progression of renal damage was considered typical for diabetic renal disease [11].

NHE study. NHE was determined as amiloride-dependent fraction of proton efflux from acidified erythrocytes into an alkaline sodium-containing medium [12, 13]. Briefly, 100 μl of packed erythrocytes were incubated with a 1.9 ml solution containing 150 mmol/l NaCl, 1 mmol/l KCl, 1 mmol/l $MgCl_2$ and 10 mmol/l glucose at pH 6.35–6.45, with or without 500 μmol/l amiloride (Sigma Chemicals, St. Louis, Mo., USA) for 5 min at 37 °C. The anion exchanger was inhibited by 200 μmol/l 4,4 '- diisothiocyanostilbene-2,2 '-disulphonic acid (DIDS; Sigma). The pH was adjusted to 7.95–8.05 by addition of NaOH. The kinetics of H^+ efflux were examined by a pH-meter (Radiometer, Kopenhagen, Denmark).

NHE was defined as an initial velocity of the amiloride-inhibited proton efflux in the above-mentioned conditions, and calculated as $(\Delta pH - \Delta pH_{amiloride})b/mt$, where ΔpH is the initial rate of the medium acidification, in the absence and presence of amiloride, respectively, b is the buffer capacity of the medium (determined by titration of 1.9 ml of the incubation medium with HCl and NaOH solutions); m is the erythrocyte volume; and t the incubation time.

NHE measurements were carried out at enrollment, and at 5 and 8 years of follow-up. The upper limit of the 'normal' NHE levels was estimated as the mean for the healthy population [13] + 2 SD, or 200 μmol H + · 1 $cells^{-1} \cdot min^{-1}$.

Table 1. Sodium-hydrogen exchange (NHE) in diabetic patients during 8 years of follow-up (total, $n = 138$)

	At enrollment	After 5 years	After 8 years
NHE levels	212 ± 24	218 ± 11	208 ± 31

(μmol $H^+ \cdot 1\ cells^{-1} \cdot min^{-1}$
Data are mean ± 2 SD

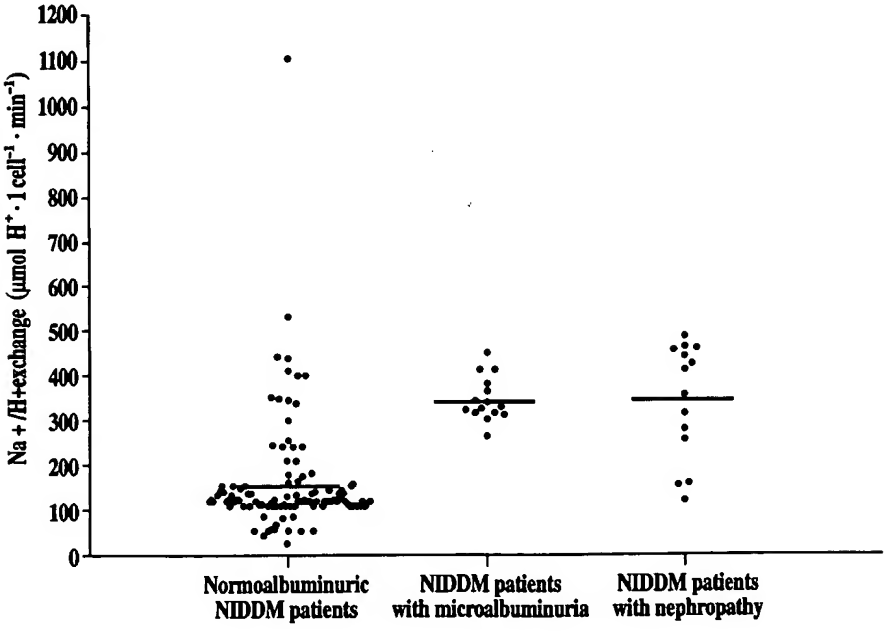

Fig. 1. Erythrocyte Na^+/H^+ exchange in normoproteinuric NIDDM patients, and NIDDM patients with microalbuminuria and nephropathy (azotaemia and/or overt proteinuria) at the end of 8 years of follow-up

Table 2. Family history of hypertension/nephropathy and rate of complications in diabetic patients with high[a] and normal NHE levels

Factors	High[a] NHE μmol H^+ · l cells^{-1} · min^{-1} 482 ± 21 (n = 51)	Normal NHE μmol H^+ · l cells^{-1} · min^{-1} 141 ± 14 (n = 87)	p value
Hypertensive relatives[b] (per patient)	1.89 ± 0.2	0.26 ± 0.1	0.01
Relatives with renal disease (per patient)	0.31 ± 0.1	0.14 ± 0.2	0.01
Fasting glucose levels (mmol/l)	10.2 ± 1.1	11.1 ± 2.0	0.68
HbA_1 (%)	12.4 ± 2.6	11.6 ± 3.1	0.66
Hypoglycaemic events per year	4.2 ± 1.1	4.6 ± 1.4	0.72
Weight gain (kg/patient)	1.6 ± 1.0	1.9 ± 0.5	0.72

Data are mean ± 2 SD
[a] High NHE = NHE > 200 μmol H^+ · l cells^{-1} · min^{-1} estimated as mean + 2 SD during the population studies [W. Koren, data not shown]
[b] First and second degree relatives, calculated as simple numbers of disease-positive relatives per patient followed-up

Statistical analysis

All values are counted as mean ± SEM. Significance was calculated by Kruskal-Wallis test for non-parametric distribution, and correlation between the values was evaluated according to Pearson Product Moment method. p values less than 0.05 were considered statistically significant.

Results

NHE was enhanced in erythrocytes of 51 (37%) patients with NIDDM, and correlated with systolic, diastolic and mean blood pressure (r = 0.38, 0.51, and 0.54, respectively, $p < 0.05$ for each). NHE did not differ between men and women of similar age and disease duration. The kinetic characteristics of the antiporter remained stable during the years of follow-up (Table 1).

During the 8 years of follow-up, one (0.7%) patient developed microalbuminuria, two (1.5%) patients had elevated MAP levels, one (0.7%) patient had established arterial hypertension, and four (2.9%) patients had elevated plasma urea and creatinine levels as a single symptom. Eight (5.8%) patients were diagnosed with microalbuminuria and elevation of MAP, and six (4.4%) patients with microalbuminuria had established arterial hypertension. Two (1.5%) patients had elevated MAP and elevated plasma urea/creatinine levels, three (2.2%) had established hypertension and azotaemia, while five (3.7%) patients consecutively passed the documented phases of elevation of MAP, established hypertension, overt proteinuria and azotaemia. All five patients with overt proteinuria met the criteria of diabetic renal disease. Proteinuria did not reach the nephrotic range in any patient. In total, 15 (11%) patients had microalbuminuria, and 14 (10.2%) patients developed overt nephropathy. Background (non-proliferative) 42 (31%) patients and 54 (40%) patients had proliferative retinopathy.

Elevated MAP appeared when diabetes had been present for 12.4 ± 1.1 years, earlier than development of microalbuminuria (18.4 ± 2.1 years, p = 0.03). Despite some overlap between the groups, the erythrocyte NHE of patients with nephropathy (azotaemia and/or overt proteinuria, n = 14) and microalbuminuria (n = 15) was significantly higher than in non-nephropathic patients with NIDDM (n = 109, Fig. 1). Elevated NHE levels were observed more frequently in patients with a family history of hypertension and renal disease (Table 2).

During the 8-year follow-up, the predictive values of elevated MAP, microalbuminuria and enhanced NHE in erythrocytes were low (Table 3). However, the sensitivity of these markers increased from elevated MAP through microalbuminuria to elevated NHE levels, and reached 93% when elevated erythrocyte NHE was associated with a positive family history of hypertension and nephropathy, or elevated MAP. Microalbuminuria was the most specific and the most accurate test the predicting nephropathy in NIDDM: specificity 92%, accuracy 86%.

There was no association between the three markers and either proliferative or background retinopathy.

The glycaemic control criteria were similar between the patients with relatively high (n = 45) and relatively low (n = 93) NHE levels, as was the range

Table 3. The comparative accuracy of MAP elevation[a], microalbuminuria and enhanced erythrocyte Na^+/H^+ exchange in predicting diabetic nephropathy[b] in patients with NIDDM (total, n = 138)

Condition	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
Elevation of mean arterial pressure[a]	0.57	0.87	0.33	0.14	0.84
Microalbuminuria	0.36	0.92	0.33	0.08	0.86
Elevated NHE	0.86	0.74	0.27	0.36	0.75
Accuracy of high NHE test associated with positive family history of hypertension/nephropathy or elevated mean arterial pressure levels[a]					
Nephropathy (total)[b]	0.93	0.59	0.20	0.69	0.62

[a] Elevated mean arterial pressure levels = mean arterial pressure > 115 mm Hg
[b] Nephropathy (total) defined as azotaemia and/or overt proteinuria

of hypoglycaemic events, weight gain and incidence of ketosis (Table 2).

Discussion

Elevation of MAP, enhancement of Na^+/H^+ exchange and microalbuminuria were simultaneously evaluated in this study as independent predictors of diabetic nephropathy. Association between the markers of nephropathy and retinopathy was not confirmed by our results, probably because 8 years of follow-up was not sufficient time to assess the relatively slow clinical course of these complications in NIDDM. The third angiopathic disorder of diabetes, neuropathy, was not assessed in this study because of technical problems.

All three determinants had a low predictive value, presumably because of low prevalence of overt nephropathy in NIDDM during the 8 years of follow-up. Enhanced erythrocyte NHE in patients with a positive family history for hypertension and/or nephropathy was the most sensitive predictor of impending renal damage: 93 with 7% rate of false-negative results. Microalbuminuria was the most specific predictor of nephropathy: 92% with 8% false-positive rate (Table 3). Thus, erythrocyte NHE study appears to be a useful method for screening of NIDDM patients who are likely to develop renal damage.

Microalbuminuria has a low sensitivity as a screening test, and develops relatively late in the course of NIDDM, after NHE enhancement and MAP elevation. Its high specificity, however, makes it useful for further evaluation of an individual diabetic patient during follow-up.

Elevated MAP increases the sensitivity of erythrocyte NHE study in NIDDM (Table 3), and might be helpful in routine assessment of NIDDM patients.

Erythrocyte NHE appeared to remain stable during the years of follow-up (Table 1). It did not vary according to the levels of glycaemic control or common diabetic complications, but did correlate with predisposition to hypertension (Table 2). The latter observation suggests a role for NHE as a marker of the clinically observed association between diabetes and hypertension [14].

The precise mechanism of NHE activation in NIDDM remains obscure. NHE enhancement in essential hypertension is generally explained by genetic factors [15, 16]. Elevated NHE rates in diabetic patients prone to nephropathy might be mediated by a similar genetic mechanism. The following evidence drawn from the present study argues in favour of this hypothesis:

(a) NHE level is an early, if not the earliest, sign among the 'predictors' of nephropathy (e.g. MAP elevation and microalbuminuria).

(b) NHE remains constant over a follow-up of several years.

(c) NHE correlates with individual tendency to arterial hypertension, and patients with enhanced NHE have an increased frequency of first and second degree relatives with hypertension and nephropathy.

NHE measurements were carried out in this study on human erythrocytes. Since the permease of Na^+/H^+ exchange is ubiquitously expressed in plasma membranes of all studied human cells [15], any cell type can be potentially be used for NHE assessment. Erythrocytes are a good and economic model of the ubiquitous membrane processes. They have the major advantages of lack of genomic and rapid post-transcriptional changes in the ion fluxes, and minimal interference between the external and intracellular membranes because of reduced number of cell compartments [16].

The NHE assay employed in this study was limited by the pH_i set-point chosen at 6.35–6.45, i.e. near pH_i for K_m [17]. Under these conditions, the maximal velocity of NHE (V_{max}) remained unassessed, but some important inferences could be made about the regulating mechanism of the NHE enhancement.

NHE is believed to depend on membrane turnover of phosphoinositides and to reflect the activation state of protein kinase C (calcium-dependent serine kinase), as well as insulin-activated tyrosine kinase [18]. Diabetic cells in vitro are characterized by increased membrane fraction of protein kinase C [19], probably due to excessive phosphoinositide turnover, enhanced calcium mobilization, and rapid phosphorylation of the cell proteins [20]. Both impaired translocation of protein kinase C [21] and overstimulated tyrosine kinase can be responsible for NHE activation in NIDDM. Protein kinase C increases the exchanger affinity for H^+_i and the NHE velocity at pH_i 6.5 (which corresponds to K_m) [22], whereas insulin fails to affect the NHE affinity of H^+_i sites [23]. Thus, the enhanced NHE in NIDDM patients with nephropathy might be attributed to impaired functioning of protein kinase C rather than to any effect of insulin.

References

1. DCCT Research Group (1995) Adverse effects and their association with treatment regimens in the Diabetes Control and Complications Trial. Diabetes Care 18: 1415–1427
2. Chavers BM, Bilous RW, Ellis EN et al. (1989) Glomerular lesions and urinary albumin excretion in type 1 diabetes without overt proteinuria. N Eng J Med 320: 966–970
3. Selby JV, FitzSimmons SC, Newman JM, Katz PP (1990) The natural history and epidemiology of diabetic nephropathy. JAMA 263: 1954–1960
4. Mogensen CE, Christensen CK, Vittinghus E (1983) The stages in diabetic nephropathy: with emphasis on the stage

of incipient diabetic nephropathy. Diabetes 32[Suppl 2]:64–78
5. Herman WH, Prior DE, Yassine MD, Weder AB (1993) Nephropathy in NIDDM is associated with cellular markers for hypertension. Diabetes Care 16: 815–818
6. Davies JE, Ng LL, Kofoed-Enevoldsen A, Li KL et al. (1992) Intracellular pH and Na^+/H^+ antiport activity of cultured skin fibroblasts from diabetics. Kidney Int 42: 1184–1190
7. Huot SJ, Aronson PS (1991) Na^+/H^+ exchanger and its role in essential hypertension and diabetes mellitus. Diabetes Care 14: 521–535
8. Unger RH, Foster DW (1992) Diabetes mellitus. In: JD Wilson, DW Foster (eds) William's textbook of endocrinology, 8th edn. Saunders, Philadelphia, pp 1255–1260
9. The 1988 Report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (1988) Arch Intern Med 148: 1023–1038
10. Chavers BM, Simonson J, Michael AF (1984) A solid phase fluorescent immunoassay for the measurement of human urinary albumin. Kidney Int 25: 576–578
11. Wass JA, Watkins PJ, Dische FE, Parsons V (1978) Renal failure, glomerular disease and diabetes mellitus. Nephron 21: 289–296
12. Escobales N, Canessa M (1986) Amiloride-sensitive Na^+ transport in human erythrocytes: evidence for a Na^+/H^+ exchange system. J Membr Biol 90: 21–28
13. Orlov SN, Postnov IY, Pokudin NI et al. (1989) Na^+/H^+ exchange and other ion-transport systems in erythrocytes of essential hypertensives and spontaneously hypertensive rats: a comparative analysis. J Hypertension 7: 781–788
14. Viberti GC, Messent J (1991) Risk factors for renal and cardiovascular disease in diabetic patients. Cardiology 79[Suppl 1]:55–61
15. Lifton RP, Sardet C, Pouyssegur J, Lalouel JM (1990) Cloning of the human genomic amiloride-sensitive Na^+/H^+ antiporter gene, identification of genetic polymorphisms and localization on the genetic map of chromosome 1p. Genomics 7: 131–135
16. Postnov IYV (1990) An approach to the explanation of cell membrane alteration in primary hypertension. Hypertension 15: 332–337
17. Semplicini A, Spalvins A, Canessa M (1989) Kinetics and stoichiometry of the human erythrocyte Na^+/H^+ exchanger. J Membr Biol 107: 219–228
18. Grinstein S, Rothstein A (1986) Mechanisms of regulation of the Na^+/H^+ exchanger. J Membr Biol 90: 1–12
19. Inoguchi T, Battan R, Handler E et al. (1992) Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycaemic control by islet cell transplantation. Proc Natl Acad Sci USA 89: 11059–11063
20. Ishii H, Umeda F, Hashimoto T, Nawata H (1990) Changes in phosphoinositide turnover, Ca2 + mobilization, and protein phosphorylation in platelets from NIDDM patients. Diabetes 39: 1561–1568
21. Nagy K, Levy J, Grunberger G (1991) Impaired translocation of protein kinase C activity in human non-insulin-dependent diabetes mellitus. Metabolism 40: 807–813
22. Canessa M, Morgan K, Goldszer R et al. (1991) Kinetic abnormalities of the red blood cell sodium-proton exchange in hypertensive patients. Hypertension 17: 340–348
23. Pontremoli R, Zerbini G, Rivera A et al (1994) Insulin activation of red blood cell Na^+/H^+ exchange decreases the affinity of sodium sites. Kidney Int 46: 365–375